in notable changes in the spectrum full width at half maximum (FWHM). We discuss these new effects in terms of the mechanism of plasmonic enhancement.

[1]Metal-Enhanced Fluorescence, Edited by Geddes, C.D., John Wiley and Sons, New Jersey, June 2010, 625 pgs, ISBN: 978-0-470-22838-8.
[2] Spectral Shifts in Metal-Enhanced Fluorescence, Karolin, J. and Geddes, C.D., (2014), *Applied Physics Letters*, 105, 063102.

3144-Pos Board B574

Ultrasensitive Detection Allows for Singlet Oxygen Phosphorescence Detection, an Important Prerequisite for Photodynamic Therapy

Marcelle Koenig[1], Manoel Veiga[1], Sebastian Tannert[1], Felix Koberling[1], Volker Buschmann[1], Matthias Patting[1], Marcus Sackrow[1], Michael Wahl[1], Rainer Erdmann[1], Peter Kapusta[2], Christian Wolf[3], Christian Kaufmann[3], Humberto Rodriguez[3].
[1]PicoQuant GmbH, Berlin, Germany, [2]J. Heyrovsky Institute of Physical Chemistry, Prague, Czech Republic, [3]PVcomB/Helmholtz-Zentrum Berlin für Materialien und Energie GmbH, Berlin, Germany.

Detection sensitivity from the ultraviolet to the near infrared spectral region is a key parameter to meet today's demand for handling smallest analyte amounts and short measurement times in the optical evaluation of miscellaneous samples. The introduction of single photon counting based data acquisition has proven to yield a major sensitivity increase and very high dynamic range - it is the ideal method for measuring weak luminescence.
We present the hardware and handling optimization of a state of the art spectrometer for steady-state and time-resolved fluorescence measurements. The high sensitivity of the spectrometer was shown by measurements of popular fluorescent dyes as well as the Raman spectrum of water under well defined and reproducible conditions. The achieved sensitivity allows us to quantify singlet oxygen generation and to characterize the singlet oxygen phosphorescence decay, a prerequisite when studying photosensitisers like porphyrins and phthalocyanines used for example in photodynamic therapy (PDT). Moreover, with the help of an integrating sphere fluorescence quantum yields of low fluorescent samples like Ru(bpy)3 in water can be determined very precisely. The fibre connection of the spectrometer to a time-resolved fluorescence microscope (MicroTime100/200) was also realized. The combination of the advantages of both setups makes it e.g. possible to perform 2D-lifetime imaging with a freely tunable detection window for low luminescent samples even far into the near infrared region. The measurements with such a combination give not only the spectral and lifetime information of a luminescent sample but also the spatial information which is especially important for hetergeneous samples.

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Accounting for Photophysical Processes and Specific Signal Intensity Change in Fluorescence-Detected Sedimentation Velocity Analytical Ultracentrifugation

Huaying Zhao[1], Jia Ma[2], Maria Ingaramo[2], Eric Andrade[2], Jeff MacDonald[3], Glen Ramsay[3], Grzegorz Piszczek[4], George Patterson[2], Peter Schuck[2].
[1]NIBIB, National Insitutes of Health, Bethesda, MD, USA, [2]NIBIB, National Institutes of Health, Bethesda, MD, USA, [3]Aviv Biomedical Inc, Lakewood, NJ, USA, [4]NHLBI, National Institutes of Health, Bethesda, MD, USA.

Fluorescence detected sedimentation velocity (FDS-SV) analytical ultracentrifugation has emerged as a powerful technique for the study of macromolecular interactions, particularly high-affinity protein interactions, with hydrodynamic resolution exceeding that of diffusion-based techniques, and with sufficient sensitivity for binding studies at low picomolar concentrations. In order to fit the FDS data structure, in the quantitative analysis it is essential to adjust the conventional sedimentation models for detailed description of the sedimentation boundaries. A key consideration is the change in the macromolecular fluorescence intensity during the course of the experiment, caused by slow drifts of the excitation laser power, and/or by photophysical processes. In the present work we demonstrate that FDS-SV data have inherently a reference for the time-dependent macromolecular signal intensity, resting on a geometric link between boundary migration and plateau signal. We show how this new time-domain can be exploited to study molecules exhibiting photobleaching and photoactivation. This expands the application of FDS-SV to proteins tagged with photoswitchable fluorescent proteins, organic dyes, or nanoparticles, such as those recently introduced for sub-diffraction microscopy. At the same time, we find conventional fluorophores undergo minimal photobleaching under standard illumination in the FDS. These findings support the application of a high laser power density for the detection, which we demonstrate can further increase the data quality.

3146-Pos Board B576

Sedimentation Velocity Analysis of the EGFPs in E. coli Whole Cell Extracts using Fluorescence Detection System

Jia Ma, Huaying Zhao, Peter Schuck.
NIBIB, NIH, Bethesda, MD, USA.

The analytical ultracentrifugation (AUC) is a classic biophysical instrument to analyze protein interactions in solutions. A recently introduced fluorescence detection system (FDS) improves the specificity and sensitivity of the AUC, and offers the potential to analyze the protein interactions in biological fluids. We explore challenges posed by the application of FDS-AUC to the study of protein interactions in *E.coli* whole cell extracts, using fluorescent proteins (EGFPs) as model system. At experimentally feasible concentrations of cell extracts, we find no discernable effects of hydrodynamic nonideality on the sedimentation of EGFPs. However, at high concentrations *E.coli* whole cell extracts produce significant signals from auto fluorescence with complicated quenching patterns. Goal of this work is to establish detection limits and develop procedures to improve specificity.

3147-Pos Board B577

Two-Color Imaging using Spectral Variants of iRFP670 and iRFP682 Near-Infrared Fluorescent Proteins

Mikhail Baloban, Daria M. Shcherbakova, Vladislav V. Verkhusha.
Anatomy and Structural Biology and Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY, USA.

The new class of fluorescent proteins (FPs) engineered from bacterial phytochromes (BphPs) attracts much attention for in vivo imaging due to their near-infrared (NIR) fluorescence spectra. These FPs utilize widely available in mammalian cells biliverdin (BV), a product of heme degradation, as a chromophore and, therefore, are as easy to use as common GFP-like proteins. We recently reported five NIR FPs, called iRFPs, with different fluorescence and biochemical properties. Interestingly, two of them, iRFP670 and iRFP682, exhibited the twice higher molecular brightness, as well as the blue-shifted absorbance (643 nm and 663 nm) and fluorescence (670 nm and 682 nm) compared to other iRFPs. Here we characterized the unusual properties of these NIR FPs in detail. Our biochemical and biophysical analysis showed that iRFP670 and iRFP682 incorporate the BV chromophore in two distinct confirmations. A single amino acid mutation resulted in a depletion of one BV confirmations in the protein binding pocket and, consequently, in 30 nm red-shifts of both absorbance and fluorescence. The point mutation also caused a slight decrease in the molecular brightness and an increase in the pH stability of the obtained red-shifted variants, which we named iRFP670-red and iRFP682-red. The effective brightness of the iRFP670-red and iRFP682-red in live mammalian cells was comparable to that of the parental proteins, suggesting that the high efficiency and high specificity of the incorporation of endogenous BV chromophore was not affected. Spectrally resolvable fluorescence of the iRFP670 and iRFP670-red pair, as well as of the iRFP682 and iRFP682-red pair, allowed easy separation of two cellular populations using FACS cytometry and straightforward two-color fluorescence microscopy of live cells, thus making them the probes of choice for cell labeling in the NIR region.

3148-Pos Board B578

Tuning the Photophysical Properties of the Green Fluorescent Protein with Unnatural Amino Acids

Gregory M. Olenginski, Christine M. Phillips-Piro, Scott H. Brewer.
Chemistry, Franklin and Marshall College, Lancaster, PA, USA.

Green fluorescent protein (GFP) is a widely utilized protein in imaging due to its favorable optical properties. These photophysical properties have previously been modified through site-directed mutagenesis utilizing naturally occurring amino acids. Here, we have further modulated the photophysical properties of GFP in a systematic fashion by the site-specific incorporation of unnatural amino acids (UAAs) into this protein. UAAs greatly enhance the ability to modulate the optical properties of this protein in a controlled manner. Optical data on these new GFP constructs containing UAAs will be presented along with initial crystallographic data relating structural changes in the protein to changes in the optical properties.

3149-Pos Board B579

Use of the Methyl Ester of a Fluorescent Unnatural Amino Acid to Facilitate Site-Specific Incorporation of Fluorescent Probes in Proteins

Joshua R. Berlin[1], William Lopez[1], Mohit R. Jain[2], Jorge E. Contreras[1].
[1]Pharmacology and Physiology, Rutgers-New Jersey Medical School, Newark, NJ, USA, [2]Center for Advanced Proteomic Research, Rutgers-New Jersey Medical School, Newark, NJ, USA.

Fluorescent unnatural amino acids (UAAs) can be efficiently incorporated in target proteins *in vivo* by expressing suppressor tRNA/aminoacyl-tRNA